AD_______________

Award Number: DAMD17-02-1-0498

TITLE: Investigation of the Lobular Carcinoma in Situ, Using Molecular Genetic Techniques, for the Involvement of Novel Genes

PRINCIPAL INVESTIGATOR: Teresa L. Mastracci
Irene L. Andrulis, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai Hospital
Toronto, Ontario, Canada M5G 1X5

REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

20050927 065

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual Summary (20 May 02 - 19 Apr 05)

4. TITLE AND SUBTITLE
Investigation of the Lobular Carcinoma in Situ, Using Molecular Genetic Techniques, for the Involvement of Novel Genes

5. FUNDING NUMBERS
DAMD17-02-1-0498

6. AUTHOR(S)
Teresa L. Mastracci
Irene L. Andrulis, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai Hospital
Toronto, Ontario, Canada M5G 1X5

E-Mail: teresam@mshri.on.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for Public Release; Distribution Unlimited	

13. ABSTRACT (Maximum 200 Words)

Atypical lobular hyperplasia (ALH) and lobular carcinoma *in situ* (LCIS), i.e. lobular neoplasia (LN), are lesions of significance in terms of risk to the patient in the development of invasive carcinoma. A correlation between the lobular histological type and inactivation of E-cadherin, a cell adhesion protein, has been reported. As well, mutations in CDH1 have been reported in invasive lobular carcinoma (ILC) and LCIS with adjacent ILC. Our study proposes to investigate LN lesions, lacking any adjacent invasive carcinoma, for alterations in and expression of known and novel genes/proteins with the goal of characterizing a molecular genetic profile. We have accrued 36 cases containing ALH/LCIS without adjacent invasive carcinoma. Previous studies have found negative E-cadherin, beta- and alpha-catenin protein expression in these lesions. Moreover, LCIS but not ALH cases were characterized by mutations and LOH at 16q was found to be an infrequent event. Recent studies have also demonstraed cytoplasmic (rather than membrane) localization of p120-catenin in LN lesions. As a mechanism for the inactivation of E-cadherin has yet to be elucidated in LN, we have used CGH microarrays to study 12 ALH and 14 LCIS lesions. Following validation by Real Time PCR it will be possible to describe events occurring at the earliest stages of breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Lobular carcinoma in situ, genetic profiling, Chromosomal CGH, CGH Microarray, Immunohistochemistry, E-cadherin	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Lobular neoplasia (LN) is a histological classification that includes atypical lobular hyperplasia (ALH) and lobular carcinoma *in situ* (LCIS). These breast lesions are of epithelial origin and histologically show a proliferative gradation from ALH to LCIS. Although a finding of LN is usually incidental during breast tissue biopsy, the relative risk to the patient in the development of invasive breast cancer is noteworthy. In fact, a number of epidemiological studies have reported that ALH and LCIS lesions are high-risk indicators, conferring a respective 4 to 5 and 8 to 10 fold increase risk.[1-3]

Previous work by our group (reported in Annual Summary Reports for 2002-2003 and 2003-2004) and others, investigated these lesions with respect to E-cadherin, a cell adhesion protein found on the membrane of epithelial cells.[4-16] Membrane E-cadherin protein expression, as well as expression of beta- and alpha-catenin, have been found to be completely and simultaneously lost in ALH, LCIS and invasive lobular carcinoma (ILC). In studies investigating ILC, the loss of expression has been explained by the acquisition of inactivating mutations in the E-cadherin gene (CDH1) coupled with loss of heterozygosity (LOH) at the chromosome locus of 16q (where CDH1 is located). However our study, specifically investigating ALH and LCIS lesions not in the presence of an invasive cancer, was unable to provide an explanation for the loss of E-cadherin protein expression in the context of CDH1 mutation and LOH at 16q.

Body

Please note that the writing of this report is based on the revised SOW submitted to USARMC early in 2005. A copy of this SOW has been appended to the report (Appendix 1).

Our studies of lobular neoplastic lesions have used the cases collected through the Mount Sinai Hospital Pathology Department. These cases were accrued with the assistance of Dr. Frances O'Malley and as of April 2005 case accrual has concluded (Task 2a). All cases in the collection are formalin-fixed, paraffin-embedded archived breast tumor blocks containing lobular neoplastic lesions lacking adjacent invasive carcinoma and, at completion, the collection includes 36 cases of ALH/LCIS.

In previous work, we established that although LCIS lesions are characterized by mutation it is likely that a mechanism other than mutation/LOH is the cause of the loss of E-cadherin protein expression in early lobular neoplastic lesions, especially in cases containing ALH. Therefore it is necessary to evaluate other mechanisms by which CDH1 may be inactivated. To this end, evaluation of methylation of the CDH1 promoter had been proposed (Task 3c, 3d). However, optimization of the methylation-specific PCR (MSP) technique[17] was unsuccessful. MSP requires a large quantity of sodium bisulfite modified DNA which was unable to be obtained from our cases of ALH/LCIS. The MethyLight technique [18,19] was also attempted because it had greater sensitivity and required less DNA template. However despite these attributes, efficient and reproducible amplification of the target promoter area was unable to be optimized, again attributed to the DNA template. Given that there are no other template options for the cases in our collection (i.e. DNA from frozen tissue), this study has been discontinued.

The optimized protocol for p120-catenin immunohistochemistry (IHC) and staining of the ALH/LCIS cases with this antibody was previously reported to be complete (Task 5a-c, Annual Report 2003-2004). Subsequently, the scoring system has been developed to evaluate the IHC for p120-catenin (Task 5d). The scoring system created assesses not only a lack of circumferential membrane staining but also the cytoplasmic localization of the protein. All LCIS lesions showed no membrane but diffuse cytoplasmic staining, as well as 10 of 11 ALH lesions, with only one case (A7) showing positive circumferential membrane staining. Together with the previously reported IHC results for E-cadherin, beta-catenin and alpha-catenin, we can conclude that without the presence of an invasive lesion the expression of the entire E-cadherin complex at the cell membrane is altered in both ALH and LCIS lesions.

Understanding the picture outside of the E-cadherin complex could be key to discerning what is altered in these early breast lesions. For this we had proposed using comparative genomic hybridization (CGH) microarrays (Task 4) to determine what other known or novel genes are altered at the lobular neoplastic stage. The collection of cases able to be studied by array-CGH (aCGH) includes 17 ALH and 15 LCIS lesions. For each lesion, four to six 8uM sections were microdissected by either laser capture microdissection or stereomicroscope-based needle dissection and DNA was extracted (Task 4a). The DNA was labeled with Cy3 and aCGH successfully performed on 12 ALH and 14 LCIS (using a female genomic DNA labeled with Cy5 as reference) (Task 4b). Each array was scanned (Applied Precision Arrayworx CCD scanner) and then the array images were loaded into the analysis software (Applied Precision Softworx) and overlaid with the spot grid. The raw data was exported from this program and subsequently visualized using SeeGH software.[20]

All arrays were analyzed using SeeGH and subsequently statistically analyzed[21] to determine if the regions found were significant (Task 4c). The statistical analysis revealed regions of alteration also found by SeeGH. In general, a greater number of alterations were found in the ALH lesions compared to the LCIS. Regions specific to ALH or LCIS are identifiable, however, there appear to be no regions that are common to the paired lesions (cases containing both ALH and LCIS). Real Time PCR is currently being optimized to validate some areas of amplification found by aCGH (Task 4d). Primer/probe sets specific to genes at 1p11.2, 10p15.2, and 20q13.13 are being assessed.

Training Experiences (2004-2005)

Teresa Mastracci is receiving her research training in the laboratory of Dr. Irene Andrulis at the Fred A. Litwin Centre for Cancer Genetics in the Samuel Lunenfeld Research Institute (SLRI) of Mount Sinai Hospital. As with any laboratory, Dr. Andrulis' lab has had turnover in the last year with the graduation of students and the acquisition of new associates. However, the laboratory continues to provide a rich research environment with 1 research associate, 2 postdoctoral fellows, 3 research technicians, 2 M.Sc. and 5 Ph.D. students. Moreover, the schedule of weekly lab meetings as well as monthly meetings with other groups in the Centre for Cancer Genetics has been maintained.

The Department of Laboratory Medicine and Pathobiology at the University of Toronto, to which Teresa is affiliated, requires the completion of 5 courses for students in the PhD program; a requirement that was fulfilled this year. Furthermore, the Department has maintained regular weekly Research Seminars which feature distinguished scientists from outside and within Toronto as well as weekly Departmental Student Seminars which give students the opportunity to present their research and receive input from the staff about their studies.

Both the University and SLRI hold yearly retreats where students and faculty present their work in a relaxed environment, facilitating discussion between laboratory groups. Teresa has presented at both of these events this year.

The Student Supervisory Committee monitors a student's progress regularly during his/her graduate career. It consists of the supervisor plus at least two other members of the Department, one of who should be from an area that is outside of the supervisor's immediate area of expertise. Committee meetings for Teresa have been consistently held every six months.

Dr. Andrulis' laboratory has also recently become involved in a weekly Molecular Medicine journal club held at the Hospital for Sick Children in Toronto. In addition, Teresa was invited to attend and present at the weekly Work In Progress Seminar Series hosted by Dr. Tak Mak at the Breast Cancer Research Institute at Princess Margaret Hospital. In the past, Teresa also has had the opportunity to attend and present at international conferences and this year has been invited to give a platform presentation at the Era of Hope DOD BCRP Meeting.

Key Accomplishments (2004-2005)

- Case accrual has been completed. The collection includes 36 cases of ALH/LCIS.

- Optimization of a methylation specific PCR protocol was unsuccessful due to the limited amount of DNA able to be obtained from each ALH/LCIS case and therefore this study has been discontinued.

- Twelve ALH and 14 LCIS lesions were successfully arrayed using the tiling BAC array generated in the laboratory of Dr. Wan Lam.[22] The results from these microarrays have been analyzed and regions specific to only ALH or LCIS cases have been identified. Real Time PCR is currently being optimized to validate some of these areas including 1p11.2, 10p15.2, and 20q13.13.

- A scoring system for p120-catenin has been developed assessing cases for a lack of membrane staining as well as cytoplasmic localization of protein. The trend for LN cases is cytoplasmic, and not membrane, localization of p120-catenin in all ALH and LCIS.

Reportable Outcomes (2004-2005)

- ***Papers*** (Appendix 3)
  Mastracci TL, Tjan S, Bane AL, O'Malley FP, Andrulis IL. E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma *in situ.* Mod Pathol. 2005 Jan 14; [Epub ahead of print]

- *Abstracts* (Appendix 2)

  Mastracci TL, Tjan S, Shadeo A, Colby S, Bane AL, Bull S, Lam W, O'Malley FP, Andrulis IL. Investigation of lobular neoplasia, using molecular genetic techniques, for the involvement of novel genes. Era of Hope Department of Defense Breast Cancer Research Program Meeting, 2005.

- ***Presentations***

  Characterization of a molecular genetic profile for lobular neoplasia. Graduate Student Research Day, Department of Laboratory Medicine and Pathobiology, University of Toronto. March 2005

  Lobular Neoplasia: an Array of discovery. Work-In-Progress Seminar Series, Breast Cancer Research Institute, Princess Margaret Hospital, Toronto. February 2005

  Profiling Lobular Neoplasia. Annual Retreat, Samuel Lunenfeld Research Institute, Geneva Park Conference Center, Orillia. October 2004

- ***Development of a Tissue Repository***

  No further cases have been added to the collection in 2004-2005 and the accrual of lobular neoplastic cases has concluded with a total of 36 ALH/LCIS cases.

Conclusions

ALH and LCIS lesions, without the presence of adjacent invasive disease, both show loss of the entire E-cadherin protein complex. Although we have shown that LCIS lesions are characterized by mutations, the genetic hits of mutation and loss of heterozygosity cannot explain this loss of E-cadherin protein expression (as well as the related loss of beta-, alpha- and p120-catenin). With the use of CGH microarray, we have found that ALH lesions appear to have a greater number of genetic alterations compared to LCIS lesions. Although many of these alterations are common to both neoplastic lesions, there appear to be regions that are specific to only ALH or LCIS. As the specific areas of amplification and deletion are currently being validated we cannot speculate yet as to the specific genetic events occurring in these lesions. However once complete, this study will define a genetic signature for lobular neoplasia that clearly describes events occurring at the earliest stages of breast cancer.

REFERENCES

1. Page DL, Schuyler PA, Dupont WD, Jensen RA, Plummer WD, Jr., Simpson JF: Atypical lobular hyperplasia as a unilateral predictor of breast cancer risk: a retrospective cohort study. Lancet 2003, 361:125-129
2. London SJ, Connolly JL, Schnitt SJ, Colditz GA: A prospective study of benign breast disease and the risk of breast cancer. Jama 1992, 267:941-944
3. Page DL, Kidd TE, Jr., Dupont WD, Simpson JF, Rogers LW: Lobular neoplasia of the breast: higher risk for subsequent invasive cancer predicted by more extensive disease. Hum Pathol 1991, 22:1232-1239.
4. Mastracci TL, Tjan S, Bane AL, O'Malley F P, Andrulis IL: E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma in situ of the breast. Mod Pathol 2005
5. Berx G, Cleton-Jansen AM, Strumane K, de Leeuw WJ, Nollet F, van Roy F, Cornelisse C: E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. Oncogene 1996, 13:1919-1925.
6. Gamallo C, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, Cano A: Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. Am J Pathol 1993, 142:987-993.
7. Moll R, Mitze M, Frixen UH, Birchmeier W: Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. Am J Pathol 1993, 143:1731-1742.
8. Goldstein NS, Bassi D, Watts JC, Layfield LJ, Yaziji H, Gown AM: E-cadherin reactivity of 95 noninvasive ductal and lobular lesions of the breast. Implications for the interpretation of problematic lesions. Am J Clin Pathol 2001, 115:534-542.
9. Goldstein NS, Kestin LL, Vicini FA: Clinicopathologic implications of E-cadherin reactivity in patients with lobular carcinoma in situ of the breast. Cancer 2001, 92:738-747.
10. Rieger-Christ KM, Pezza JA, Dugan JM, Braasch JW, Hughes KS, Summerhayes IC: Disparate E-cadherin mutations in LCIS and associated invasive breast carcinomas. Mol Pathol 2001, 54:91-97.
11. Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, van Roy F: E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. Embo J 1995, 14:6107-6115.
12. Huiping C, Sigurgeirsdottir JR, Jonasson JG, Eiriksdottir G, Johannsdottir JT, Egilsson V, Ingvarsson S: Chromosome alterations and E-cadherin gene mutations in human lobular breast cancer. Br J Cancer 1999, 81:1103-1110.
13. Vos CB, Cleton-Jansen AM, Berx G, de Leeuw WJ, ter Haar NT, van Roy F, Cornelisse CJ, Peterse JL, van de Vijver MJ: E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis. Br J Cancer 1997, 76:1131-1133
14. De Leeuw WJ, Berx G, Vos CB, Peterse JL, Van de Vijver MJ, Litvinov S, Van Roy F, Cornelisse CJ, Cleton-Jansen AM: Simultaneous loss of E-cadherin and catenins in invasive lobular breast cancer and lobular carcinoma in situ. J Pathol 1997, 183:404-411.
15. Droufakou S, Deshmane V, Roylance R, Hanby A, Tomlinson I, Hart IR: Multiple ways of silencing E-cadherin gene expression in lobular carcinoma of the breast. Int J Cancer 2001, 92:404-408.
16. Sarrio D, Perez-Mies B, Hardisson D, Moreno-Bueno G, Suarez A, Cano A, Martin-Perez J, Gamallo C, Palacios J: Cytoplasmic localization of p120ctn and E-cadherin loss

characterize lobular breast carcinoma from preinvasive to metastatic lesions. Oncogene 2004, 23:3272-3283
17. Herman JG GJ, Myohanen S, Nelkin BD, Baylin SB.: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996, 93:9821-9826
18. Trinh BN, Long TI, Laird PW: DNA methylation analysis by MethyLight technology. Methods 2001, 25:456-462
19. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW: MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000, 28:E32
20. Chi B, DeLeeuw RJ, Coe BP, MacAulay C, Lam WL: SeeGH--a software tool for visualization of whole genome array comparative genomic hybridization data. BMC Bioinformatics 2004, 5:13
21. Olshen AB, Venkatraman ES, Lucito R, Wigler M: Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 2004, 5:557-572
22. Ishkanian AS, Malloff CA, Watson SK, DeLeeuw RJ, Chi B, Coe BP, Snijders A, Albertson DG, Pinkel D, Marra MA, Ling V, MacAulay C, Lam WL: A tiling resolution DNA microarray with complete coverage of the human genome. Nat Genet 2004, 36:299-303

Appendix 1: Revised Statement of Work (SOW)

MASTRACCI, TERESA L.

STATEMENT OF WORK

Investigation of lobular carcinoma *in situ,* using molecular genetic techniques, for the involvement of novel genes.

Task 1: Completion of the Analysis of E-cadherin

a. Using the previously microdissected LCIS DNA, screen the remaining exons of the E-cadherin gene using Single Strand Conformation Polymorphism (SSCP).
b. Excise alterations, appearing as shifted bands, from the SSCP gel and extract the altered DNA.
c. Manually sequence the DNA to characterize each alteration.

Task 2: Tissue Accrual

a. Collaborate with Dr. Frances O'Malley to search/request new cases of LCIS and ALH from external hospitals and institutions.
b. Using a microtome, cut newly acquired formalin-fixed, paraffin-embedded (FFPE) blocks, mount sections on glass slides, and microdissect/extract the DNA.

Task 3: Evaluation of methods of inactivation of E-cadherin

a. Using the previously microdissected DNA from all cases, evaluate loss of heterozygosity (LOH) for chromosomal region 16q using five microsatellite markers (D16S421, D16S496, D16S503, D16S3095, D16S752) and a PCR-based method to determine the LOH status of each tumor/normal pair (the PCR products separated using 7% denaturing formamide gel and visualized with autoradiography.
b. Multiple independent observers evaluate each LOH marker, scoring each case as 'LOH', 'no LOH', or 'uninformative'.
c. Optimization of methylation-specific PCR (MSP) technique using hypermethylated placenta DNA and methylated cell-line DNA, in order to assess the E-cadherin promoter for methylation.
d. Carry out MSP using DNA from all cases, and assess methylation status of the E-cadherin promoter for each case.

Task 4: Analysis of LCIS and ALH by CGH Microarray

a. Microdissect/extract DNA from all cases.
b. Carry out CGH microarray experiments using optimized protocol and tiling BAC array (DNA of interest labeled with Cy3, reference DNA labeled with Cy5).
c. Quantification and statistical analysis of microarray experiments using scanners and software available through collaboration with Dr. Wan Lam (B.C. Cancer Research Center, British Columbia, Canada) and Dr. Shelly Bull (SLRI, Toronto, ON, Canada)
d. Validation of results by Real Time PCR with custom primers.

Task 5: Analysis of LCIS and ALH by Immunohistochemistry

a. Using a microtome, cut FFPE LCIS blocks and mount sections on glass slides.
b. Optimize antibody (beta-catenin, alpha-catenin, p120-catenin) concentrations using any available FFPE breast tumor sections and FFPE cell lines.
c. Stain sections with antibodies for beta-catenin, alpha-catenin, and p120-catenin.
d. Collaborate with Dr. Frances O'Malley to evaluate the results of these immunohistochemical experiments.

Appendix 2: Abstract submitted for Era of Hope DOD BCRP Meeting, 2005.

INVESTIGATION OF LOBULAR NEOPLASIA, USING MOLECULAR GENETIC TECHNIQUES, FOR THE INVOLVEMENT OF NOVEL GENES

Teresa L. Mastracci(1,2), Suzanna Tjan(3), Ashleen Shadeo(4), Sarah Colby(5), Anita L. Bane(1,2,3), Shelley Bull(5), Wan Lam(4), Frances P. O'malley(2,3), Irene L. Andrulis(1,2)

1 Samuel Lunenfeld Research Institute, Fred A. Litwin Centre for Cancer Genetics, Mount Sinai Hospital, Toronto, Ontario, Canada;
2 Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada;
3 Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada;
4 B.C. Cancer Research Centre, Department of Cancer Genetics and Developmental Biology, Vancouver, British Columbia, Canada;
5 Samuel Lunenfeld Research Institute, Prosserman Centre for Health Research, Mount Sinai Hospital, Toronto, Ontario, Canada.

teresam@mshri.on.ca

Breast cancer is one of the most prevalent human cancers and a leading cause of death among women. Lobular neoplasia is a classification of breast lesions that includes atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS). Both ALH and LCIS are premalignant lesions that are impalpable and mammographically silent. Though these lesions are only found incidentally during breast biopsy, ALH/LCIS lesions are significant in terms of implication of risk to the patient in the development of invasive carcinoma. A strong correlation between the lobular histological subtype and inactivation of E-cadherin, a protein involved in cell adhesion, has been reported. As well, mutations in the E-cadherin gene (CDH1) have been reported in invasive lobular carcinoma (ILC) and LCIS with adjacent ILC. We have been investigating lobular neoplastic lesions, lacking any adjacent invasive carcinoma, for alterations in and expression of known and novel genes/proteins with the goal of characterizing a molecular genetic profile for lobular neoplasia.

We have obtained 21 archived cases of which there are 13 ALH lesions and 13 LCIS lesions all without adjacent invasive carcinoma. E-cadherin, beta-, alpha-, and p120-catenin protein expression were assessed by immunohistochemistry (IHC). Sequence alterations in CDH1 were identified using Single Strand Conformation Polymorphism (SSCP)/manual sequencing and PCR-based loss of heterozygosity (LOH) analysis was carried out for the 16q locus. Following the characterization of the E-cadherin complex, array-CGH was used to determine gene copy number alterations in these cases.

By IHC, we have found negative E-cadherin (23/24), beta-catenin (23/24), and alpha-catenin (21/23) protein expression in these lesions. Cytoplasmic (rather than membrane) localization of p120-catenin was observed in 20 of 21 lesions. LCIS cases were characterized by mutations, however ALH cases were not. LOH at 16q was found to be an infrequent event. Array-CGH has been carried out on 11 LCIS and 9 ALH lesions and the data is currently being analyzed to determine the molecular genetic profiles.

Loss of the E-cadherin adhesion complex is an early event in lobular neoplasia affecting ALH as well as LCIS and occurs prior to progression to invasive disease. However, loss of E-cadherin protein expression is accompanied by DNA alterations in LCIS but not in ALH. Cases lacking both protein expression and gene alterations suggest that another mechanism is involved, possibly as early as at the hyperplastic stage, causing the silencing of the E-cadherin complex. Moreover, the array-CGH study may allow us to characterize a molecular genetic profile for lobular neoplasia, identifying events that mark the earliest stages in the development of cancer. This advance will significantly expand our ability to design strategies for the prevention of invasive carcinoma and could identify targets for therapeutic treatment.

The U.S. Army Medical Research and Materiel Command under DAMD17-02-1-0498 supported this work.

Modern Pathology (2005) 1–11

www.modernpathology.org

E-cadherin alterations in atypical lobular hyperplasia and lobular carcinoma *in situ* of the breast

Teresa L Mastracci[1,2], Suzanna Tjan[3], Anita L Bane[1,2,3], Frances P O'Malley[2,3] and Irene L Andrulis[1,2,4]

[1]*Fred A Litwin Centre for Cancer Genetics, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada;* [2]*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada;* [3]*Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada and* [4]*Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada*

Tumor development from an early lesion through to invasive disease is not a clearly defined progression in the breast. Studies of invasive lobular carcinoma have reported mutations, loss of heterozygosity (LOH) and loss of protein expression in epithelial (E)-cadherin, a protein involved in cell adhesion. Our study examines *in situ* lobular neoplastic lesions without concurrent invasive carcinoma for E-cadherin gene alterations and protein expression, beta-catenin, alpha-catenin and p120-catenin protein expression, and LOH at the chromosome 16q locus, with the goal of determining the events occurring at the stage of lobular neoplasia. In all, 13 atypical lobular hyperplasia lesions and 13 lobular carcinoma *in situ* lesions from archived cases were examined. E-cadherin sequence alterations were evaluated using single strand conformation polymorphism and DNA sequencing, and PCR-based LOH analysis was carried out for the 16q locus. Using immunohistochemistry, we assessed protein expression. A total of 23 of 24 lesions evaluated by immunohistochemistry were negative for both E-cadherin and beta-catenin protein expression, and 21 of 23 lesions were negative for alpha-catenin. Cytoplasmic (rather than membrane) localization of p120-catenin was observed in 20 of 21 cases. Lobular carcinoma *in situ* cases were characterized by mutations; however, atypical lobular hyperplasia cases were not. LOH at 16q was an infrequent event. From our study, we conclude that an altered E-cadherin adhesion complex is an early event affecting atypical lobular hyperplasia as well as lobular carcinoma *in situ* and occurs prior to progression to invasive disease. However, the loss of protein expression is accompanied by E-cadherin DNA alterations in lobular carcinoma *in situ* but not in atypical lobular hyperplasia. These cases lacking both protein expression and gene alterations suggest that another mechanism is involved, possibly as early as at the hyperplastic stage, causing silencing of the E-cadherin complex.
Modern Pathology advance online publication, 14 January 2005; doi:10.1038/modpathol.3800362

Keywords: atypical lobular hyperplasia; breast cancer; E-cadherin; lobular carcinoma *in situ*; lobular neoplasia

Tumor development from an early lesion through to invasive disease is not a clearly defined progression in the breast. For invasive breast carcinomas of the lobular histological subtype there is increasing evidence that *in situ* lobular neoplastic lesions are not only indicators of increased risk but may also act as precursors in the progression to invasive carcinoma.[1] Our study investigates *in situ* lobular neoplastic lesions that have not progressed to invasive disease with the goal of determining the molecular genetic events occurring at the stage of lobular neoplasia. Discovering the specific events that mark the transition from an early lobular neoplastic lesion to an invasive tumor is necessary to both support and subsequently understand this breast cancer progression.

Lobular neoplasia is a histological classification that includes atypical lobular hyperplasia and lobular carcinoma *in situ*. Lobular carcinoma *in situ* is a lesion of epithelial origin and is defined by a population of cells that are small, round, monomorphic and discohesive. The lesion is often

Correspondence: Dr IL Andrulis, PhD, Fred A Litwin Centre for Cancer Genetics, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., room 984, Toronto, Ontario, Canada M5G 1X5.
E-mail: andrulis@mshri.on.ca
Received 27 September 2004; revised and accepted 5 November 2004; publishd online 14 January 2005

multicentric and bilateral, and greater than 50% of the acini in the affected terminal duct lobular unit are distended by the cellular proliferation. The cells that define atypical lobular hyperplasia are similar to those that characterize lobular carcinoma *in situ*, however, the cellular proliferation does not completely occlude the lumen, and less than 50% of the acini in the affected terminal duct lobular unit exhibit distension.[2] Both lobular neoplastic lesions are found incidentally during breast tissue biopsy due to their inability to be detected by palpation or mammography.

Histologically, there is a proliferative gradation from lobular hyperplasia to *in situ* carcinoma, which is also reflected in the relative risk to the patient in the development of invasive disease. A number of epidemiological studies have reported that lobular neoplastic lesions are high-risk indicators.[3–5] A finding of atypical lobular hyperplasia has been reported to imply a four- to five-fold increased risk of subsequent carcinoma in either breast, and a finding of lobular carcinoma *in situ* implies an eight- to ten-fold increased risk to the patient.

In sporadic breast cancers, histological type has been correlated with expression of the cell adhesion protein epithelial (E)-cadherin, the cadherin subtype expressed in epithelial cells. Proteins that complex with E-cadherin at the cell membrane include beta-, gamma-, alpha- and p120-catenin. Reduced expression of E-cadherin has been reported in invasive ductal carcinoma whereas lobular carcinoma *in situ* and invasive lobular carcinoma show complete loss of the protein.[6–16] A report on invasive lobular carcinomas with adjacent lobular carcinoma *in situ* demonstrated not only loss of E-cadherin expression but also the simultaneous loss of beta-, gamma- and alpha-catenin protein expression.[15] More recently, Sarrio *et al*[17] demonstrated that the loss of E-cadherin along with the cytoplasmic localization of p120-catenin characterizes lobular breast cancers and suggested that p120-catenin plays a role in mediating the oncogenic effects of E-cadherin loss in these cancers. Unquestionably there is evidence that an altered E-cadherin adhesion complex is characteristic of invasive lobular carcinoma and lobular carcinoma *in situ* with adjacent invasive lobular carcinoma.

In light of these findings, several studies have investigated the E-cadherin gene, *CDH1*, for alterations in lobular carcinomas. Mutations have been detected in invasive lobular carcinoma and lobular carcinoma *in situ* with adjacent invasive,[6,11–16] and the loss of chromosome 16q was detected in solitary *in situ* and synchronous *in situ*/invasive lesions.[1,18] These investigations of lobular carcinoma and E-cadherin have provided some evidence that *in situ* lobular carcinoma may be not only a risk indicator but also a precursor lesion to invasive carcinoma.

To date, most molecular genetic studies of lobular carcinoma *in situ* have focused on lesions with adjacent invasive carcinoma. However, to study events occurring specifically at the hyperplastic and *in situ* stages, it is necessary to examine cases where the neoplastic lesion is not contaminated by an invasive lesion. Cases containing atypical lobular hyperplasia and lobular carcinoma *in situ* without adjacent invasive carcinomas are known to occur in only 0.5–3.8% of breast cases that are otherwise benign.[5,19] These lesions have rarely been studied at the molecular level. We have accrued a collection of cases fitting these criteria, making it possible to determine protein expression and gene alterations occurring at the stage of lobular neoplasia.

Materials and methods

Tissue Accrual

The study population consisted of 21 formalin-fixed, paraffin-embedded, archived cases accrued through the Mount Sinai Hospital Pathology Department (Toronto, ON, Canada). The cases were accessioned from 1988 to 2003. The study pathologist (FOM) reviewed each neoplastic lesion from hematoxylin and eosin (H&E)-stained sections. Previously described histological characteristics[20] were used to classify the lobular lesions. The collection included 13 atypical lobular hyperplasia lesions (A1–A13) and 13 lobular carcinoma *in situ* lesions (L1–L12) lacking adjacent invasive carcinoma. Four of these cases contained both hyperplasia and *in situ* lesions (A2/L1, A3/L3, A4/L5, A11/L12). More specifically, case A2/L1 was also characterized by multifocal lobular carcinoma *in situ* (L1-1, L1-2) and these *in situ* lesions were housed in separate blocks.

Two cases of interest, supplementary to our collection, were also included in all analyses. Case A14/L13 contained atypical lobular hyperplasia and lobular carcinoma *in situ* lesions as well as a focus of invasive lobular carcinoma in a separate block; the lobular neoplastic lesions were analyzed. Moreover, case P1 contained a lesion of low-grade ductal carcinoma *in situ* that was used as a control for the immunohistochemistry analyses. All cases were coded to prevent bias and maintain confidentiality.

To determine the robustness of the histological classification of the lesions, all cases were reviewed independently by a second pathologist (ALB) blinded to the results of the molecular analyses. There were discrepancies in two cases. Both cases had been called lobular carcinoma *in situ* by the first pathologist (FOM) and 'atypical lobular hyperplasia with duct involvement by cells of atypical lobular hyperplasia' by the second pathologist (ALB). A consensus diagnosis of lobular carcinoma *in situ* was reached for both cases by the pathologists following rereview at a multiheaded microscope. Ultimately, the pathology review confirmed that our collection contained 13 atypical lobular hyperplasia and 13 lobular carcinoma *in situ* lesions without adjacent invasive carcinoma.

Microdissection, DNA Extraction and DNA Amplification

From each case, serial sections (8 μm) were cut from the block containing the lesion of interest and mounted on glass slides. Following deparaffinization, the lesion was removed from the section using either a stereomicroscope-based microdissection technique[21] or laser-capture microdissection (Pix-Cell II, Arcturus, CA, USA). The microdissection technique used was determined by the size of the lesion. The majority of the *in situ* lesions (L1-1, L1-2, L2, L4, L6–L11) were microdissected using the stereomicroscope-based technique due to large lesion area. However, all hyperplasia lesions (A1–A14) were small in size and required laser-capture microdissection in order to accurately remove the lesion from the surrounding tissue. Cases L3, L5, L12 and L13 were also microdissected by laser-capture microdissection because they contained both neoplastic lesions in close proximity in the same section. Figure 1 depicts a case containing atypical lobular hyperplasia from our collection and demonstrates the lesion before and after laser-capture microdissection, as well as the degree of cellularity of a typical atypical lobular hyperplasia lesion for our collection. Whether by stereomicroscope or laser-capture, the use of a microdissection technique allowed for the isolation of a population of lobular neoplastic cells from each atypical lobular hyperplasia or lobular carcinoma *in situ* lesion that was assured to contain no greater than 15–20% contamination of non-neoplastic cells. Following microdissection, the tissue was incubated for 48 h and DNA was extracted using the QiaAMP DNA Mini Kit (Qiagen, Canada).

As neoplastic lesions are small in size, the amount of DNA available per case was limited. To increase the quantity of DNA available for mutation detection, we used the whole genome amplification technique degenerate oligonucleotide-primed polymerase chain reaction (DOP PCR)[22] Random degenerate oligonucleotide primers and a modified PCR cycle were used to amplify the added DNA template. Each DOP PCR reaction contained 2 μl of microdissected DNA template and was amplified as per the manufacturer's instructions (DOP PCR Kit, Roche Biomolecular, Canada).

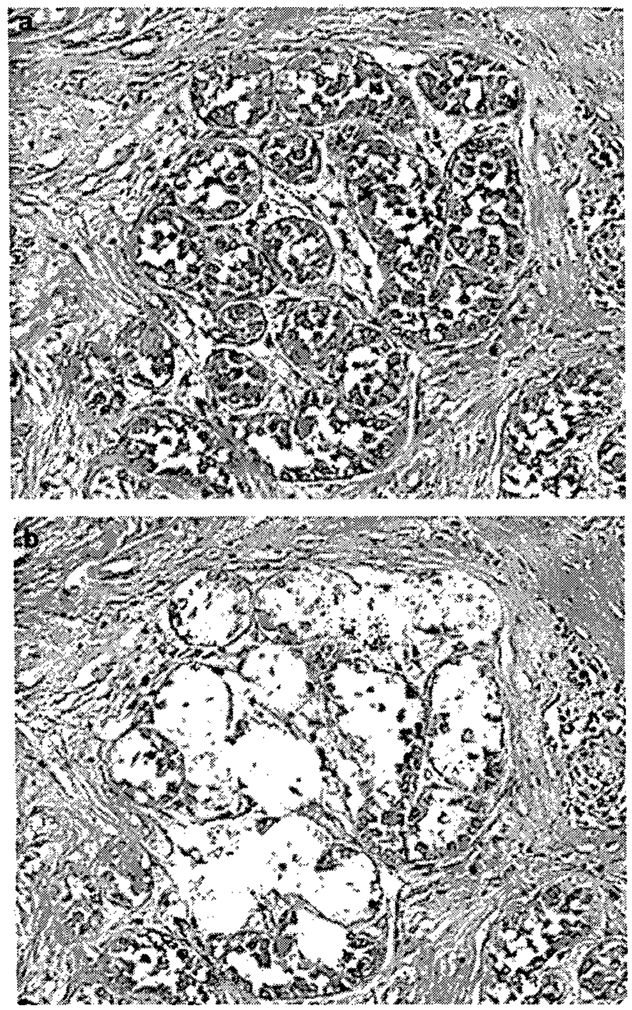


Figure 1 An atypical lobular hyperplasia lesion from our collection before and after laser-capture microdissection. (a) A section containing atypical lobular hyperplasia, stained with hematoxylin, before microdissection by the laser-capture microdissection technique. (b) The tissue remaining on the slide following microdissection of the lesion. Using laser-capture microdissection we could maintain the purity of the lesions, with only 15–20% contamination by non-neoplastic cells (intermediate power, $\times$ 20).

Sequence Alteration Detection and Characterization

To detect sequence alterations, we used single strand conformation polymorphism (SSCP) followed by manual DNA sequencing to characterize each alteration. For the initial screening, each exon of *CDH1* was individually amplified using PCR. Exon-specific PCR conditions were optimized for all primer pairs (exons 1–3, 6–16[6]; exons 4–5[11]). Amplification was performed in a volume of 30 μl containing 10 μl of DOP PCR product template, 1 $\times$ High Fidelity PCR Buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.3 μM of forward and reverse primers, 1 U of PLATINUM Taq DNA polymerase High Fidelity (GIBCO BRL, Life Technologies, Canada). and 0.1 μCi of ^{33}P(Phosphorus-33)-labeled dATP (Perkin-Elmer, USA). Following an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 15 s, 50°C (exons 2, 3, 7–10, 16), or 53°C (exon 6), or 55°C (exons 4 and 5), or 58°C (exons 11–15), or 68°C (exon 1) for 15 s, and 72°C for 20 s were performed.

A stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to the SSCP reactions, which were subsequently heat denatured and subjected to electrophoresis on an 8% nondenaturing polyacrylamide gel (including 10% glycerol). Two gels were run simultaneously under different conditions: (i)

4°C, 8 W for 12 h, and (ii) room temperature, 12 W for 16 h. Results were obtained following autoradiography. If an aberrantly migrating band was observed, the SSCP reaction was repeated for that case using 5 μl of microdissected DNA template that had not been subjected to DOP PCR amplification (PCR/SSCP conditions as previously stated). If the abnormal banding pattern could be duplicated in this second independent SSCP experiment, then the alteration was characterized using manual DNA sequencing.

To characterize *CDH1* alterations, aberrantly migrating bands were excised from the dried SSCP gel and DNA was extracted using a serial freeze–thaw technique. In all, 5 μl of the extracted DNA was used as template in an exon-specific PCR reaction using the conditions previously outlined. The Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Canada) was used to manually sequence the DNA, according to the manufacturer's instructions. Results were obtained following autoradiography. Alterations that were found were confirmed by repeating the sequencing using microdissected DNA (not previously preamplified by DOP PCR) as template from both the forward and reverse direction for each exon.

Loss of Heterozygosity (LOH)

LOH was evaluated for the E-cadherin gene located on chromosome 16q. Five microsatellite markers, located at chromosome locus 16q21–16q22.1, were used (D16S421, D16S496, D16S503, D16S3095, D16S752). Microdissected DNA from each lesion (not preamplified by DOP PCR), paired with DNA from an adjacent area of normal tissue, was used as template to examine LOH.

PCR amplification was performed in a volume of 30 μl containing 5 μl of microdissected DNA template, 1 × High Fidelity PCR Buffer, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.3 μM of forward and reverse primers, 1 U of PLATINUM Taq DNA polymerase High Fidelity (GIBCO BRL, Life Technologies, Canada). and 0.1 μCi of [^{33}P]dATP (Perkin-Elmer, USA). Following an initial denaturation step at 94°C for 3 min, 40 cycles of 94°C for 15 s, 54°C (D16S503) or 57°C (D16S421, D16S496, D16S752, D16S3095) for 15 s, and 72°C for 20 s were performed. A stop solution (as previously described) was added and each reaction was subsequently heat denatured and subjected to electrophoresis on a 7% denaturing formamide gel, which was run at 80 W for 3 h. Results were obtained following autoradiography. Multiple independent observers evaluated each marker, scoring each case as 'LOH', 'no LOH', or 'uninformative'. To evaluate each marker, LOH was defined as a relative decrease in band intensity greater than 50%. For each case, a minimum of three of the five markers with observed LOH was required for an overall classification of LOH.

Immunohistochemistry

Following sectioning for microdissection, 4 μm sections were cut for each formalin-fixed, paraffin-embedded block, and mounted on glass slides. Each section was deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Following heat antigen retrieval, the primary antibodies to E-cadherin (HECD-1, Monoclonal Mouse anti-E-cadherin 2nd Gen Predilute Antibody, Zymed Laboratories Inc., USA), beta-catenin (Monoclonal Mouse anti-beta-catenin, 1:6000 dilution, Transduction Laboratories, USA), alpha-catenin (NCL-A-CAT, Monoclonal Mouse anti-alpha-catenin, 1:50 dilution, Novocastra Laboratories Ltd, UK) or p120-catenin (p120-ctn (15D2), 1:50 dilution, Santa Cruz Biotechnology, Inc., USA) were applied. The Ultra Streptavidin Detection System (Signet Laboratories Inc., USA) was used as per the manufacturer's instructions for all antibodies except alpha-catenin, which required the ELITE Detection System (Vector Laboratories (Canada) Inc., Canada). Each section was developed with the chromogen diaminobenzidene and sections were counterstained in hematoxylin.

Some cases have insufficient material to carry out immunohistochemistry due to sectioning order. Sections were cut from each case for immunohistochemistry analyses only after sectioning was complete for microdissection. In some cases, the lesion of interest was no longer present in the immunohistochemistry section and therefore no result could be obtained.

Immunohistochemical staining was reviewed and scored by the study pathologist. To evaluate the immunohistochemistry for E-cadherin, beta-catenin and alpha-catenin protein expression, a positive stain was determined to be complete circumferential membrane staining of the lobular neoplastic cells. The case of low-grade ductal carcinoma *in situ* (P1) was used as a positive control for E-cadherin, beta-catenin and alpha-catenin staining as it expressed these proteins at the membrane. Evaluation of the immunohistochemistry for p120-catenin required assessment of the circumferential membrane staining as well as the cytoplasmic staining pattern. A formalin-fixed, paraffin-embedded breast cancer cell line (MCF7) was used as the positive control for the p120-catenin immunohistochemistry as it contained membrane localized p120-catenin.

Results

CDH1 Mutation Analysis

Using the manual DNA sequencing technique, 16 polymorphisms (data not shown) and 15 mutations (Table 1) were characterized. Three mutations (cases A12, L4, L8) were deletions causing a frameshift and a premature stop codon. In all, 11 sequence alterations were classified as missense mutations (cases

Table 1 Summary of *CDH1* mutation, immunohistochemistry and LOH results

Case accrual		*Mutation analysis*			*Immunohistochemistry*[a]				*LOH*
Case	*Lesion*	*Alteration*	*Exon*	*Effect*	*E-cad*	*β-cat*	*α-cat*	*p120-cat*	
A1	ALH	None			–	–	–	cyto	No
A2(L1)	ALH	None			–	–	–	cyto	No
A3(L3)	ALH	None			–	–	–	cyto	NR
A4(L5)	ALH	None			–	–	–	cyto	No
A5	ALH	None			–	–	–	cyto	No
A6	ALH	None			NR	NR	NR	NR	NR
A7	ALH	None			+	+	+	+	No
A8	ALH	None			–	–	–	cyto	No
A9	ALH	None			–	–	–	cyto	No
A10	ALH	None			–	–	–	cyto	Yes
A11(L12)	ALH	None			–	–	–	NR	No
A12	ALH	2410delC	15	Frameshift	–	–	–	cyto	No
A13	ALH	None			–	–	–	cyto	No
L1-1(A2)	LCIS	856G>A	7	Ala>Thr	–	–	–	cyto	No
L1-2(A2)	LCIS	362A>G	3	His>Arg	–	–	–	cyto	Yes
L2	LCIS	274C>T	3	His>Tyr	–	–	–	cyto	No
L3(A3)	LCIS	2125G>A	13	Ala>Thr	–	–	–	cyto	No
L4	LCIS	1323_1333del	10	Frameshift	–	–	–	cyto	No
L5(A4)	LCIS	1366G>A	10	Val>Met	–	–	–	cyto	No
L6	LCIS	1676G>A	11	Ser>Asn	–	–	–	cyto	No
L7	LCIS	185G>A	3	Gly>Asp	–	–	–	cyto	Yes
L8	LCIS	1309_1310del	9	Frameshift	–	–	+	NR	No
L9	LCIS	760G>T	6	Asp>Tyr	–	–	NR	cyto	NR
L10	LCIS	989C>T	7	Thr>Ile	–	–	–	cyto	No
L11	LCIS	2075C>T	13	Ala>Val	NR	NR	NR	NR	No
L12(A11)	LCIS	1800A>G	12	Ile>Met	–	–	–	NR	No
A14(L13)[b]	ALH	None			–	–	NR	NR	No
L13(A14)	LCIS	1595G>A	11	Trp>amber	–	–	NR	NR	No
P1[c]	DCIS	None			+	+	+	NR	NR

ALH = atypical lobular hyperplasia; LCIS = lobular carcinoma *in situ*; DCIS = ductal carcinoma *in situ*; (–), negative membrane staining; (+), positive membrane staining; NR = no result due to insufficient material; cyto = diffuse cytoplasmic staining.

[a]All cases contained adjacent normal breast acini that served as the internal positive control for the immunohistochemical analyses.

[b]Case A14/L13 contained atypical lobular hyperplasia and lobular carcinoma *in situ* lesions as well as a focus of invasive lobular carcinoma in a separate block. The lobular neoplastic lesions were analyzed.

[c]Case P1 contained a lesion of low-grade ductal carcinoma *in situ* and was used as a positive control for the E-cadherin, beta-catenin and alpha-catenin immunohistochemistry experiments as it contains no lobular neoplasia.

L1–1, L1–2, L2, L3, L5, L6, L7, L9, L10, L11, L12). The mutations were found in exons 3, 6, 7, 9, 10, 11, 12, 13 and 15. With the exception of the missense mutation found in case L1–1 (previously reported by Rieger-Christ *et al*[11]), all alterations found in this study are novel.

Four cases (A2/L1, A3/L3, A4/L5, A11/L12) contained both atypical lobular hyperplasia and lobular carcinoma *in situ* lesions. Each of these cases of lobular carcinoma *in situ* contained a sequence alteration that was not detected in the adjacent hyperplastic lesions. In addition to containing both types of lesions, case A2/L1 contained, in separate blocks, two lobular carcinoma *in situ* lesions (L1–1, L1–2). These *in situ* lesions were microdissected individually and each was found to harbor different missense mutations.

In order to predict if the missense mutations found in our cases of lobular carcinoma *in situ* would have a phenotypic effect we used SIFT (http://blocks.fhcrc.org/sift/SIFT.html), a sequence homology-based tool. Of the 11 missense mutations, three were predicted by SIFT not to be tolerated amino-acid substitutions (case L5, L9, L10).

Case A14/L13, noted as containing adjacent atypical lobular hyperplasia and lobular carcinoma *in situ* lesions as well as invasive lobular carcinoma in a separate block, was found to contain a nonsense mutation in exon 11. As observed in the four cases containing both lobular neoplastic lesions, the sequence alteration is present in the *in situ* component but not in the hyperplasia. Figure 2 shows the mutation found in L13 and the corresponding sequence from the adjacent hyperplasia (A14) lacking the alteration.

Loss of Heterozygosity

LOH was evaluated with five microsatellite markers and each case was evaluated paired with its corresponding normal for each marker (Table 1).

We observed 80–100% agreement between observers and questionable cases were repeated and reevaluated. Three cases had insufficient material to carry out LOH analysis (A3, A6, L9). Of the remaining cases, three were found to have LOH (A10, L1–2, L7) and 20 showed no LOH (Figure 2).

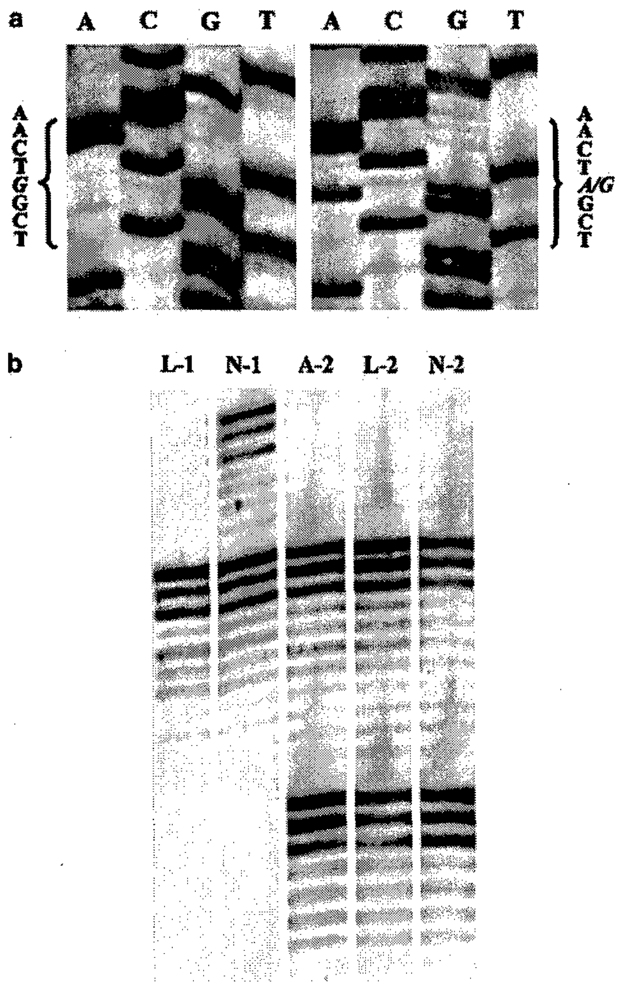


Figure 2 Characterization of *CDH1* sequence alterations and LOH at 16q. (**a**) Sequence alteration found in case A14/L13. The alteration was found in the lobular carcinoma *in situ* lesion (sequence on the right) and not the atypical lobular hyperplasia lesion (sequence on the left). The autoradiograph shows the missense mutation (1595G>A) that translates into an amino-acid change of Trp to amber, which causes a stop in the sequence in exon 11 of *CDH1*. (**b**) Case L7 (denoted as L-1, N-1) contains LOH and case A11/L12 (denoted as A-2, L-2, N-2) does not. LOH was determined using DNA from the lesions paired with adjacent normal tissue. The lesions are labeled as 'A' for atypical lobular hyperplasia, 'L' for lobular carcinoma *in situ*, and 'N' for corresponding normal epithelium.

E-cadherin, Beta-catenin, Alpha-catenin and p120-catenin Protein Expression

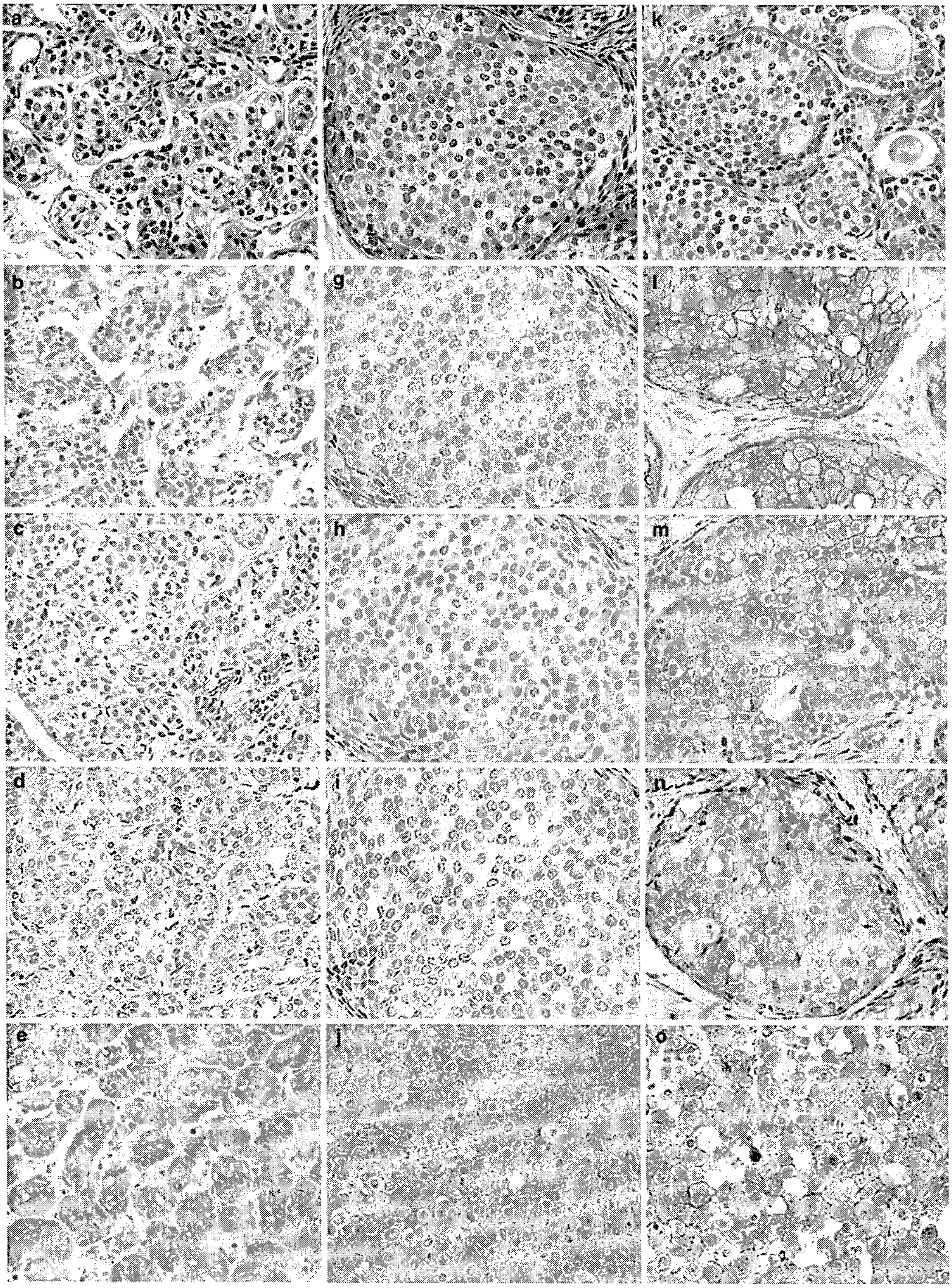
All lobular carcinoma *in situ* and 11 of 12 atypical lobular hyperplasia lesions were negative for E-cadherin and beta-catenin staining (Table 1, Figure 3); case A7 showed positive staining of the lobular neoplastic cells despite its identical morphologic appearance to the other cases of atypical lobular hyperplasia. The lesions in case A14/L13 had an identical staining pattern to all the lobular neoplastic cases, with negative E-cadherin and beta-catenin protein expression. For alpha-catenin, 10 of 11 lobular carcinoma *in situ* and 11 of 12 atypical lobular hyperplasia lesions were scored as negative, and two cases (A7, L8) were positive. The case of low-grade ductal carcinoma *in situ* (P1) stained positive for E-cadherin, beta-catenin and alpha-catenin. Moreover, all cases contained adjacent normal epithelium that served as an internal positive control and in all cases showed complete circumferential membrane staining (Figure 4).

The formalin-fixed, paraffin-embedded breast cancer cell line (MCF7) showed membrane localization of p120-catenin, and therefore positive circumferential membrane staining. Conversely, all lobular carcinoma *in situ* lesions showed no membrane, but diffuse cytoplasmic staining, as did 10 of 11 atypical lobular hyperplasia lesions, and only one case (A7) showed complete circumferential membrane staining.

Discussion

We investigated atypical lobular hyperplasia and lobular carcinoma *in situ* lesions for E-cadherin gene alterations and protein expression, beta-, alpha-, and p120-catenin protein expression, and LOH at the chromosome 16q locus. Unlike most studies, our atypical lobular hyperplasia and lobular carcinoma *in situ* cases were specifically selected without adjacent invasive carcinoma. The information to be gained from studying these lobular neoplastic lesions is substantial when considering the ambig-

Figure 3 Examination of E-cadherin, beta-catenin, alpha-catenin and p120-catenin protein expression by immunohistochemistry. (**a**) Case A2, containing atypical lobular hyperplasia, stained with H&E to show cellular architecture. The corresponding negative membrane staining for (**b**) E-cadherin (some background spotty cytoplasmic staining is observed with this antibody), (**c**) beta-catenin, (**d**) alpha-catenin and (**e**) p120-catenin stained sections from this case. The p120-catenin stain shows cytoplasmic localization of the protein. (**f**) An H&E-stained section from a case containing lobular carcinoma *in situ* (case L2); and the corresponding negative membrane staining for (**g**) E-cadherin, (**h**) beta-catenin, (**i**) alpha-catenin and (**j**) p120-catenin for this case. The p120-catenin staining of the *in situ* case shows cytoplasmic localization with some accentuation in the perinuclear zone. (**k**) An H&E-stained lesion of low-grade ductal carcinoma *in situ* (case P1), used as a positive control for the (l) E-cadherin, (m) beta-catenin, and (n) alpha-catenin immunohistochemistry experiments. (**o**) Formalin-fixed, paraffin-embedded MCF7 breast cancer cell line stained for p120-catenin and used as a positive control for p120-catenin immunohistochemistry (high power, ×40).


a
b
c
d
e
f
g
h
i
j
k
l
m
n
o

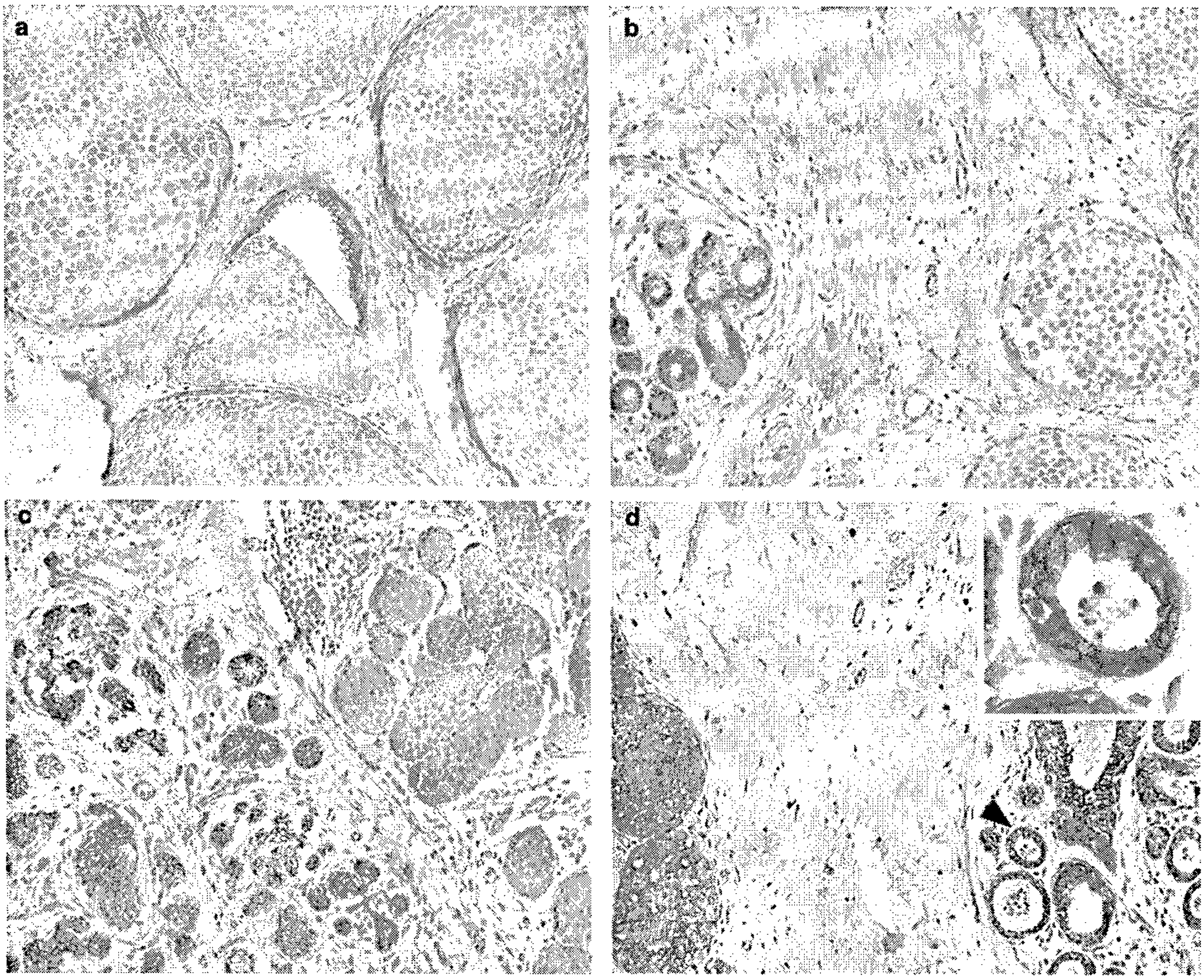


Figure 4 Normal breast acini stained by immunohistochemistry. All cases of atypical lobular hyperplasia and lobular carcinoma *in situ* contained areas of normal nonproliferating acini, adjacent to the lesion of interest, which served as internal positive control (Intermediate power, ×20). (**a**) Case L2 stained for E-cadherin protein expression, with complete circumferential membranous staining in the area of normal epithelium. (**b**) Case L2 stained for beta-catenin, showing adjacent normal acini with complete circumferential membranous staining. (**c**) Case A11/L12 stained for alpha-catenin, with adjacent normal acini showing complete circumferential membranous staining. (**d**) Case A5 stained for p120-catenin demonstrates cytoplasmic localization of the protein in the lesion and complete circumferential membranous staining in the adjacent normal acini (inset: high power, ×40).

uous understanding of the molecular genetic events occurring at these early stages.

A number of studies have shown that E-cadherin is completely inactivated in invasive lobular carcinoma. Definitively, from our study of neoplastic lesions, we can conclude that lobular lesions, whether hyperplasia or carcinoma *in situ*, lack E-cadherin membrane staining. These immunohistochemistry results support the previously reported correlation between protein inactivation and histological type. Furthermore, the data indicate that this correlation is not restricted to lobular carcinoma *in situ* and invasive lobular carcinoma but can be extended to atypical lobular hyperplasia as well.

In addition, a complete lack of beta-catenin and alpha-catenin protein expression as well as cytoplasmic localization of p120-catenin was characteristic to the lobular neoplastic cells in all but one case. We postulate that this one exceptional case (A7), with membrane localization of all proteins of the E-cadherin complex, has not yet undergone the molecular genetic events that cause inactivation of the E-cadherin complex. Irrespective of case A7, the results of our immunohistochemical analyses demonstrate that without the presence of an invasive lesion the expression of the entire E-cadherin complex at the cell membrane is altered in both atypical lobular hyperplasia and lobular carcinoma *in situ* lesions.

The use of a whole genome amplification technique to increase the quantity of the DNA template obtained from the lobular neoplastic lesions made it possible to complete the screening of *CDH1* for sequence alterations. DOP PCR has been used previously in combination with SSCP and is sensitive with respect to amplifying small quantities of DNA.[23] According to us *CDH1* mutation analysis by DOP PCR-SSCP was reliable, as aberrantly migrating bands found by using this technique could be duplicated by SSCP using microdissected DNA, without prior amplification by DOP PCR, as template.

Only one (A12) of 13 atypical lobular hyperplasia lesions contained an alteration. On the other hand, every case of lobular carcinoma *in situ* analyzed has been found to harbor a sequence alteration (13/13). To date, there have been no mutations reported in cases of lobular carcinoma *in situ* that lacked adjacent invasive carcinoma; somatic mutations have only been found in invasive lobular carcinoma or lobular carcinoma *in situ* with adjacent invasive disease. Although the data do not allow us to speculate as to whether these *in situ* lesions are precursors to invasive carcinoma, we can conclude that somatic alterations in *CDH1* appear to occur predominantly at the *in situ* stage.

The study design, the sensitivity of the techniques used and the high cellularity of the lesions of atypical lobular hyperplasia ruled out the possibility of false-negative results with respect to *CDH1* mutation analysis. The microdissection techniques allowed for the isolation of each lesion from the surrounding tissue/adjacent lesions with no greater than 15–20% contamination of non-neoplastic cells (Figure 1). In addition, the lack of sequence alterations found in the hyperplasia cases was reproduced in independent experiments using microdissected DNA (not previously preamplified by DOP PCR) from each atypical lobular hyperplasia lesion. Altogether the trend that lobular carcinoma *in situ* but not atypical lobular hyperplasia cases carry alterations was prominent. Furthermore, this trend supports previous reports of a precursor role for *in situ* lesions as it demonstrates an increase in genetic hits from hyperplasia to carcinoma *in situ* characteristic of progression.

Moreover, in the cases that contained both lobular neoplastic lesions, the atypical lobular hyperplasia and lobular carcinoma *in situ* were microdissected separately and in all cases the *in situ* component was found to harbor a sequence alteration, whereas hyperplasia did not. Even case A14/L13, noted in the pathology review as containing adjacent atypical lobular hyperplasia and lobular carcinoma *in situ* lesions as well as invasive lobular carcinoma in a separate block, was found to contain a mutation in the *in situ* stage but not hyperplasia. These cases further substantiate our hypothesis that mutations are first detected at the *in situ* stage.

CDH1 sequence alterations reported to date in studies investigating progression from lobular carcinoma *in situ* to invasive lobular carcinoma have been inactivating mutations. The nonsense mutation found in case A14/L13 was predicted to cause protein truncation. As this case (A14/L13) has an adjacent invasive lesion, we suggest that the presence of an inactivating *CDH1* mutation could be an event that distinguishes lobular carcinoma *in situ* lesions that are precursors from those that are not.

The frameshift mutations found in cases L4, L8 and A12 are likely to have an effect on protein function. The bioinformatics tool, SIFT, clarified to some extent the functional significance of the missense mutations we detected. As only three missense mutations were predicted to affect protein function, it is likely that lobular carcinoma *in situ* lesions found to harbor only missense mutations do not progress to invasive carcinoma. The presence of a missense mutation could simply indicate an environment amenable to genetic alteration, as observed in case A2/L1 where adjacent *in situ* lesions were found to harbor different missense mutations. Moreover, since the loss of protein expression was not always associated with a sequence alteration, as in the cases containing atypical lobular hyperplasia, we conclude that mutation alone could not cause the lack of E-cadherin protein expression that we have observed.

Very little is known about the molecular genetic events occurring at the stage of atypical lobular hyperplasia. To our knowledge, this study represents the first investigation of alterations in E-cadherin in atypical lobular hyperplasia. The overwhelming absence of mutations in cases of atypical lobular hyperplasia, coupled with a loss of E-cadherin protein expression, suggests that in these lesions, E-cadherin may be inactivated by means other than the presence of mutation. To address this, we evaluated these lesions for LOH.

LOH has been studied in lobular breast cancers and the chromosomal region of 16q, the location of *CDH1*,[24] has been found to have a high degree of LOH.[6,12,13,16] These previous studies have found LOH to accompany mutations in cases of invasive lobular carcinoma or lobular carcinoma *in situ* with adjacent invasive lobular carcinoma. However, in the present study, all cases of lobular carcinoma *in situ* were found to harbor mutations but LOH was found in only two of these 13 cases. In both instances, LOH was detected in cases that harbored missense mutations. The classic pattern of an inactivating mutation coupled with LOH does not appear to be characteristic of the lobular carcinoma *in situ* lesions in our collection.

Methylation of the E-cadherin promoter has been reported in studies investigating invasive lobular carcinoma,[16] breast carcinomas,[25] and breast cancer cell lines that lack E-cadherin expression.[26,27] In the case of sporadic gastric carcinomas, promoter methylation has been described as a second hit leading to inactivation of the E-cadherin gene.[28] We hypothesize that epigenetic mechanisms acting at the hyperplastic stage could provide an explanation for the loss of E-cadherin that we have observed in both atypical lobular hyperplasia and lobular carcinoma *in situ*. Further research efforts will address this possibility.

Although many epidemiological studies have clarified the risk associated with atypical lobular hyperplasia and lobular carcinoma *in situ*, at present there are no morphological or clinical features that help identify those individuals who have the greatest risk of developing invasive disease. From our study, we conclude that an altered E-cadherin adhesion complex, including alpha-, beta- and p120-catenin, is characteristic of lobular neoplastic lesions and occurs prior to progression to invasive disease. Furthermore, somatic mutations appear to be an event characteristic of lobular carcinoma *in situ* and not atypical lobular hyperplasia lesions, and we suggest inactivating mutations could possibly distinguish the lobular carcinoma *in situ* lesions that may progress to invasive disease. However, the reported molecular data, that is, mutations, LOH, chromosomal gains/losses, and loss of protein expression, coupled with the findings of our investigation, still leave questions regarding the progression from lobular neoplasia to invasive breast cancer. Further research into the molecular events occurring at the hyperplastic and *in situ* stages is essential to understanding and identifying this subset of lobular neoplastic lesions that have the highest risk of progressing to invasive carcinoma.

Acknowledgements

This work was supported by the Canadian Breast Cancer Research Initiative Grant (009177) to IL Andrulis, and the United States Army Medical Research and Material Command predoctoral fellowship (DAMD 17-02-1-0498) to TL Mastracci. We wish to thank Dr Nalan Gokgoz, Sasha Eskandarian and Nona Arneson for helpful discussions and invaluable technical support throughout these studies; the laboratory technicians in the Pathology Department at Mount Sinai Hospital; and Drs Susan J Done and Louise A Quenneville for assistance with case accrual.

References

1 Shelley Hwang E, Nyante SJ, Yi Chen Y, *et al.* Clonality of lobular carcinoma *in situ* and synchronous invasive lobular carcinoma. Cancer 2004;100:2562–2572.
2 Lishman SC, Lakhani SR. Atypical lobular hyperplasia and lobular carcinoma *in situ*: surgical and molecular pathology. Histopathology 1999;35:195–200.
3 Page DL, Schuyler PA, Dupont WD, *et al.* Atypical lobular hyperplasia as a unilateral predictor of breast cancer risk: a retrospective cohort study. Lancet 2003;361:125–129.
4 London SJ, Connolly JL, Schnitt SJ, *et al.* A prospective study of benign breast disease and the risk of breast cancer. JAMA 1992;267:941–944.
5 Page DL, Kidd Jr TE, Dupont WD, *et al.* Lobular neoplasia of the breast: higher risk for subsequent invasive cancer predicted by more extensive disease. Hum Pathol 1991;22:1232–1239.
6 Berx G, Cleton-Jansen AM, Strumane K, *et al.* E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. Oncogene 1996; 13:1919–1925.
7 Gamallo C, Palacios J, Suarez A, *et al.* Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. Am J Pathol 1993;142:987–993.
8 Moll R, Mitze M, Frixen UH, *et al.* Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. Am J Pathol 1993;143:1731–1742.
9 Goldstein NS, Bassi D, Watts JC, *et al.* E-cadherin reactivity of 95 noninvasive ductal and lobular lesions of the breast. Implications for the interpretation of problematic lesions. Am J Clin Pathol 2001;115: 534–542.
10 Goldstein NS, Kestin LL, Vicini FA. Clinicopathologic implications of E-cadherin reactivity in patients with lobular carcinoma *in situ* of the breast. Cancer 2001;92:738–747.
11 Rieger-Christ KM, Pezza JA, Dugan JM, *et al.* Disparate E-cadherin mutations in LCIS and associated invasive breast carcinomas. Mol Pathol 2001;54:91–97.
12 Berx G, Cleton-Jansen AM, Nollet F, *et al.* E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. EMBO J 1995;14:6107–6115.
13 Huiping C, Sigurgeirsdottir JR, Jonasson JG, *et al.* Chromosome alterations and E-cadherin gene mutations in human lobular breast cancer. Br J Cancer 1999;81:1103–1110.
14 Vos CB, Cleton-Jansen AM, Berx G, *et al.* E-cadherin inactivation in lobular carcinoma *in situ* of the breast: an early event in tumorigenesis. Br J Cancer 1997;76:1131–1133.
15 De Leeuw WJ, Berx G, Vos CB, *et al.* Simultaneous loss of E-cadherin and catenins in invasive lobular breast cancer and lobular carcinoma *in situ*. J Pathol 1997;183:404–411.
16 Droufakou S, Deshmane V, Roylance R, *et al.* Multiple ways of silencing E-cadherin gene expression in lobular carcinoma of the breast. Int J Cancer 2001; 92:404–408.
17 Sarrio D, Perez-Mies B, Hardisson D, *et al.* Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. Oncogene 2004;23:3272–3283.
18 Etzell JE, Devries S, Chew K, *et al.* Loss of chromosome 16q in lobular carcinoma *in situ*. Hum Pathol 2001;32:292–296.
19 Haagensen CD, Lane N, Lattes R, *et al.* Lobular neoplasia (so-called lobular carcinoma *in situ*) of the breast. Cancer 1978;42:737–769.
20 Dupont WD, Page DL. Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med 1985;312:146–151.
21 Done SJ, Arneson NC, Ozcelik H, *et al.* p53 mutations in mammary ductal carcinoma *in situ* but not in epithelial hyperplasias. Cancer Res 1998;58:785–789.
22 Telenius H, Carter NP, Bebb CE, *et al.* Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. Genomics 1992;13:718–725.

23 Barbaux S, Poirier O, Cambien F. Use of degenerate oligonucleotide primed PCR (DOP-PCR) for the genotyping of low-concentration DNA samples. J Mol Med 2001;79:329–332.
24 Berx G, Staes K, van Hengel J, *et al.* Cloning and characterization of the human invasion suppressor gene E- cadherin (CDH1). Genomics 1995;26:281–289.
25 Nass SJ, Herman JG, Gabrielson E, *et al.* Aberrant methylation of the estrogen receptor and E-cadherin 5′ CpG islands increases with malignant progression in human breast cancer. Cancer Res 2000;60:4346–4348.
26 Graff JR, Herman JG, Lapidus RG, *et al.* E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res 1995;55:5195–5199.
27 Hiraguri S, Godfrey T, Nakamura H, *et al.* Mechanisms of inactivation of E-cadherin in breast cancer cell lines. Cancer Res 1998;58:1972–1977.
28 Machado JC, Oliveira C, Carvalho R, *et al.* E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. Oncogene 2001;20: 1525–1528.